Application Note

Aggregation Analysis Using SE-HPLC and SE-UHPLC Methods in *USP* General Chapter <129>

Analytical Procedures for Recombinant Therapeutic Monoclonal Antibodies

Biologics™

Monoclonal antibodies (mAbs) represent one of the fastest growing drug markets worldwide. Since the FDA first approved an antibody therapy (Muromonab CD3) in 1986, over 100 mAbs have been granted U.S. FDA approval.

Manufacturing processes or degradation upon storage can alter the physiochemical properties of these proteins leading to formation of aggregates higher molecular weight species (HMWS). In addition to its impact on quality, the loss of product, and potential batch failure, protein aggregation has been shown to increase immunogenicity and the risk of an unintended harmful immune response, therefore making it a critical quality attribute (CQA) [1].

The biophysical characterization of mAbs is critical for approval and life cycle management. Because of the complex nature of biologics, analytical tests are often challenging and time consuming. USP has developed three analytical Reference Standards (RS) that can be used to overcome some of these challenges by providing users with well characterized standards. Additionally, USP offers a system suitability Standard that allows an analyst to easily assess system suitability criteria described in USP General Chapter <129>.

Purity assessment by size exclusion chromatography (SEC) is most commonly used to assess aggregation. This CQA is a stability-indicating attribute that is assessed throughout the lifecycle of every commercial mAb. Traditionally, size-exclusion high performance liquid chromatography (SE-HPLC) has been used for measuring monomer, high-molecular, and low-molecular weight species; however, size-exclusion high performance liquid chromatography (SE-UHPLC) is becoming the preferred method due to the advantages of UHPLC as compared to HPLC. UHPLC is an advanced separation technique that allows for shorter run times, better chromatographic separation, and increased throughput as compared to traditional HPLC. This is made possible by the higher pressure limits in a UHPLC, which allows for the use of columns with lower particles sizes. Recently, USP published a revision of General Chapter <129> *Analytical Procedures for Recombinant Therapeutic Monoclonal Antibodies* for public comment in the *Pharmacopeial Forum* (PF) *49(3)* that now includes SE-UHPLC to align with industry trends [2].

Table 1. General information for the three non-compendial USP mAb Reference Standards

	USP mAb 001,	**USP mAb 002,**	**USP mAb 003,**
USP Catalog #	1445539	1445547	1445595
MW	~147,000 Da	~150,000 Da	~146,000 Da
HMWS (%)[1]	0.9	0.8	0.4
Monomer (%)[1]	99.1	99.2	99.6
LMWS (%)[1]	<0.1	<0.1	<0.1
Package size	200 µl solution (2 mg protein content)	200 µl solution (2 mg protein content)	200 µl solution (2 mg protein content)

[1] *Purity data as reported on each USP mAb certificate [3 – 5]. All data were generated using SE-HPLC method.*

In this study, three well characterized USP mAb reference standards, USP mAb 001, USP mAb 002, and USP mAb 003, were used for SE-HPLC and SE-UHPLC testing, as per methods described in General Chapter <129>, from *PF 49(3)*. All three mAbs are recombinant humanized IgG1 isotypes expressed in CHO cell culture and produced by common industry purification processes [3 – 5]. The USP mAb reference standards have been thoroughly evaluated by SE-HPLC using the method from *USP* General Chapter <129> **(Table 1)** and other methods [6, 7]. The aim of this study was to demonstrate comparability of the two methods, SE-HPLC and SE-UHPLC using USP mAb RS.

Experimental

The method parameters, instruments, and materials are described in **Table 2**. With the SE-UHPLC method, 4x less mAb is required (50 µg per injection) as compared to the SE-HPLC method (200 µg), because columns with smaller pore size and diameter can be used with UHPLC. As stated above, one of the key advantages of UHPLC is the potential for increased throughput, which is evident by the 12 minute shorter run time for SE-UHPLC (18 minutes) as compared to SE-HPLC (30 minutes).

Table 2. SE-HPLC and SE-UHPLC Method Parameters

Method	SE-HPLC	SE-UHPLC
Sample Concentration	10 mg/mL	5 mg/mL
Mobile Phase	0.2 M potassium phosphate and 0.25 M potassium chloride, pH 6.2.	
Column Details	7.8 mm x 30 cm, 5-µm, 250 Å, packing L59	4.6 mm x 30 cm, 2-µm, 250 Å, packing L59
Column Temperature	25°C	
Gradient	Isocratic	
Flow Rate	0.5 mL/min	0.3 mL/min
Run Time	30 min	18 min
Detector	UV 280 nm	
Sampling Rate	10 points/sec	2 points/sec
Filter Time Constant	Normal (0.2000 s)	Normal (1.000 s)
Autosampler Temperature	5°C	
Injection Volume	20 µL	10 µL

The SEC system suitability requirements listed in *USP* General Chapter <129> were assessed using USP Monoclonal IgG System Suitability Reference Standard (Catalog # 1445550) [8]. For both SE-HPLC and SE-UHPLC, all system suitability passed acceptance criteria per *USP* General Chapter <129> **(Table 3)**. System suitability for both SE-HPLC and SE-UHPLC methods use USP Monoclonal IgG System Suitability RS and have identical acceptance criteria. Example chromatograms of System Suitability samples from both SE-HPLC and SE-UHPLC are shown in **Figure 1**.

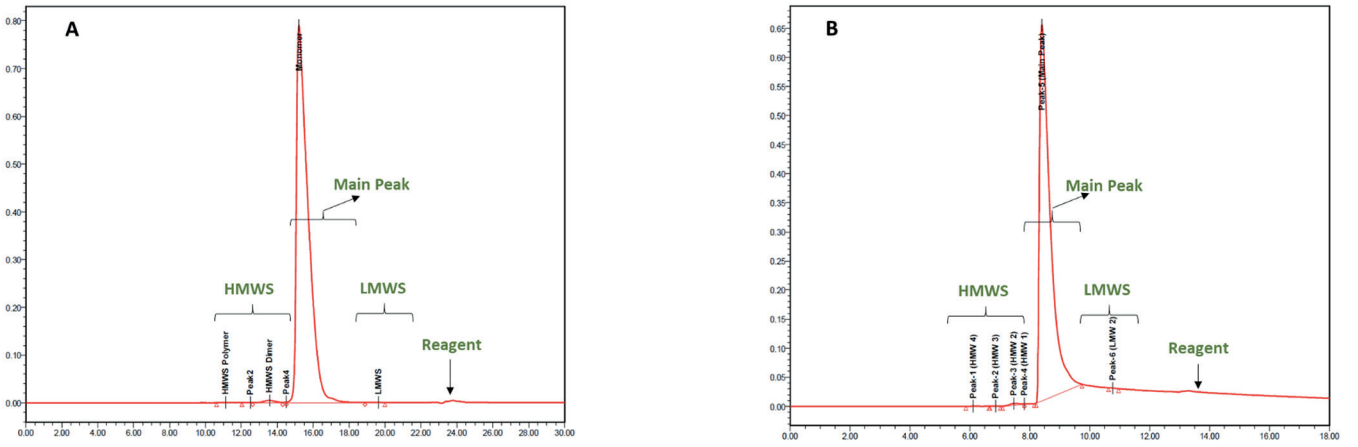

Figure 1: Representative System Suitability Chromatograms for (A) SE-HPLC and (B) SE-UHPLC with USP Monoclonal IgG System Suitability RS

Table 3. System Suitability Results using USP Monoclonal IgG System Suitability RS

Criteria	HPLC	UHPLC
The chromatographic profiles of System suitability solution injections that bracket injections of Sample solutions in a sample set are consistent with each other and with the typical chromatographic profile as illustrated in the USP Certificate for USP System Suitability RS.	Pass	Pass
All named peaks in the typical chromatogram must be present, be resolved, and in the same elution order as that in the System suitability solution chromatograms.	Pass	Pass
The percent peak area of the HMWS in the System suitability solution must be 0.4% - 0.67%	Pass	Pass
The percent peak area of the main peak in the System suitability solution must be 99.1% - 99.6%	Pass	Pass
The percent peak area of the LMWS in the System suitability solution must be ≤ 0.2%	Pass	Pass

Results

All three USP mAb reference standards have been evaluated previously by multi-laboratory studies using SE-HPLC. The main peak of USP mAb 001, USP mAb 002, and USP mAb 003 are 99.1%, 99.2%, and 99.6%, respectively, and all three have <0.1% LMWS **(Table 1)**. Here, we present the results from an internal study that included parallel testing of all three USP mAb reference standards by SE-HPLC and SE-UHPLC to compare the two methods. For each USP mAb, the percentage of the main peak (monomer), high molecular weight (HMW) impurities, and low molecular weight (LMW)impurities were determined from two injections from a single preparation. As listed in **Table 4**, both results are very close to the original values reported in the multi-lab SE-HPLC for all the three mAb standards. For both SE-HPLC and SE-UHPLC, the main peak, HMWS, and LMWS peak percentage was ±0.2% for each mAb as compared to their certificate values. Lastly, the profiles of all 3 USP mAb samples were similar when comparing SE-HPLC vs SE-UHPLC chromatograms **(Figure 2,3 & 4).**

Table 4. SE-HPLC and SE-UHPLC Results

Method	Average %Area	USP mAb 001	USP mAb 002	USP mAb 003
SE-UHPLC	HMWS	1.1	0.7	0.4
	Main Peak	98.9	99.2	99.5
	LMWS	<0.1	<0.1	0.1
	HMWS	0.7	0.9	0.4
	Main Peak	99.3	99.1	99.6
	LMWS	<0.1	<0.1	<0.1

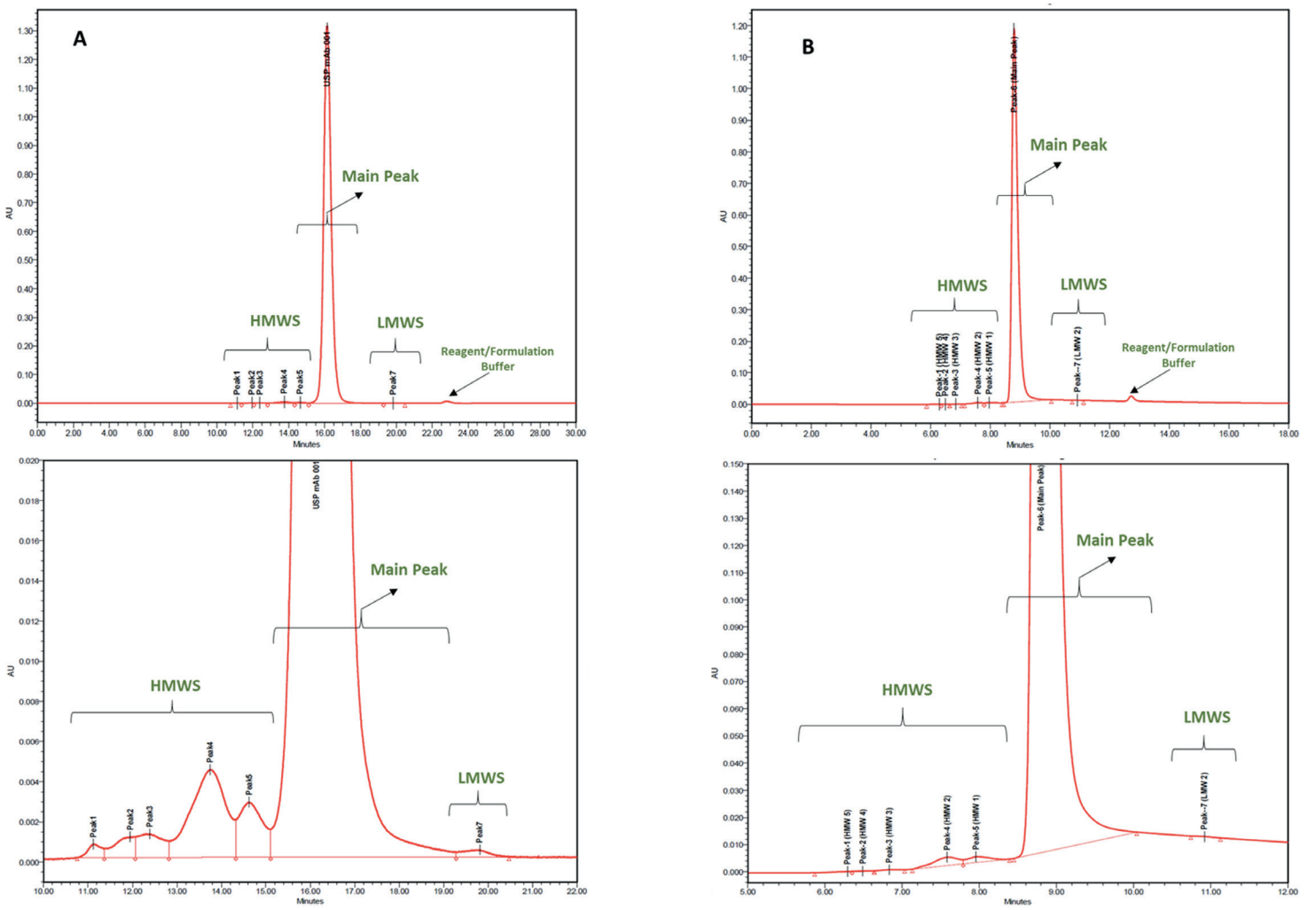

Figure 2: Representative Size Exclusion Chromatograms for USP mAb 001 Monoclonal IgG1 (A) SE-HPLC (B) SE-UHPLC. Top images show full scale while bottom images show zoomed scale.

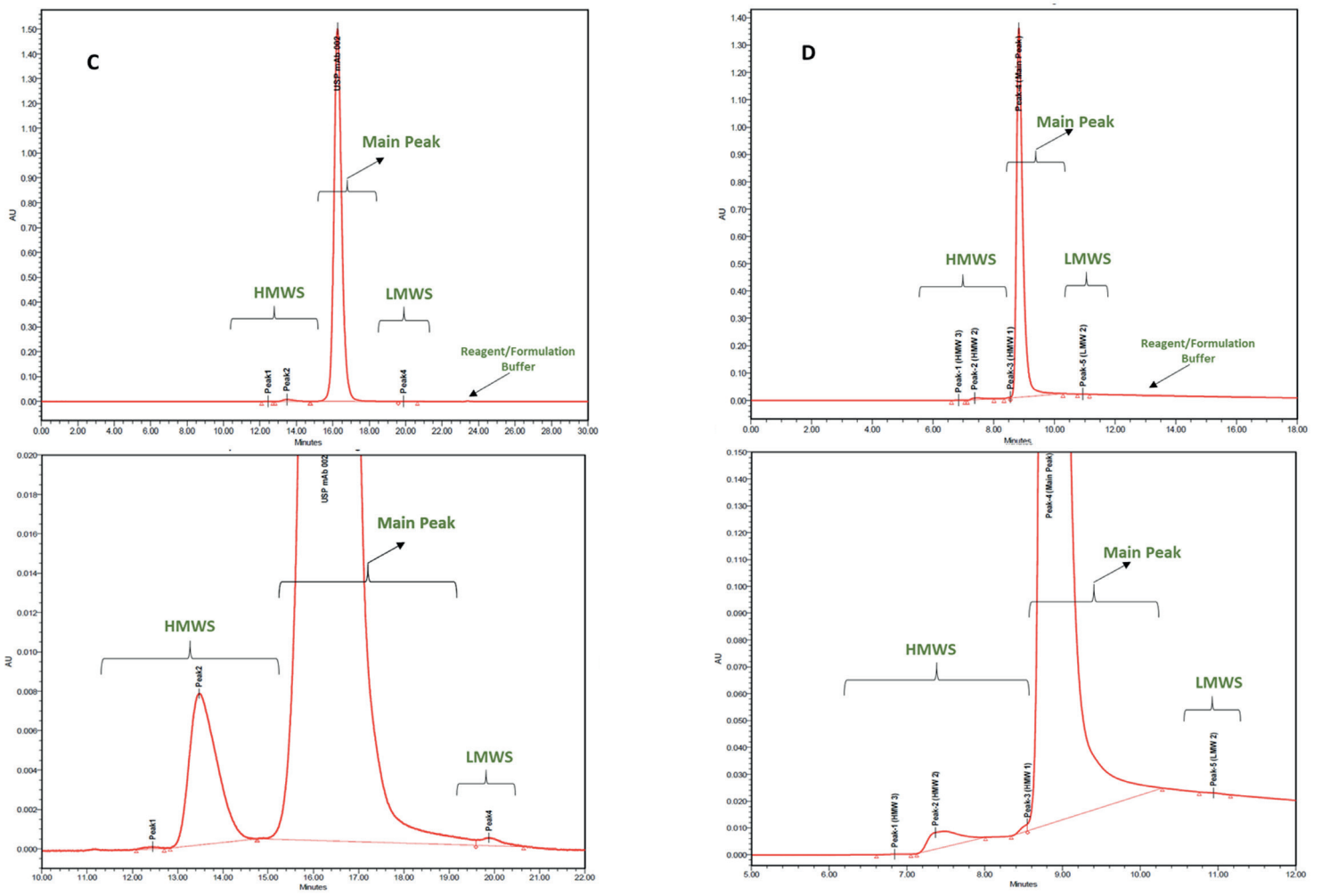

Figure 3: Representative Size Exclusion Chromatograms for USP mAb 002 Monoclonal IgG1 (C) SE-HPLC (D) SE-UHPLC. Top images show full scale while bottom images show zoomed scale.

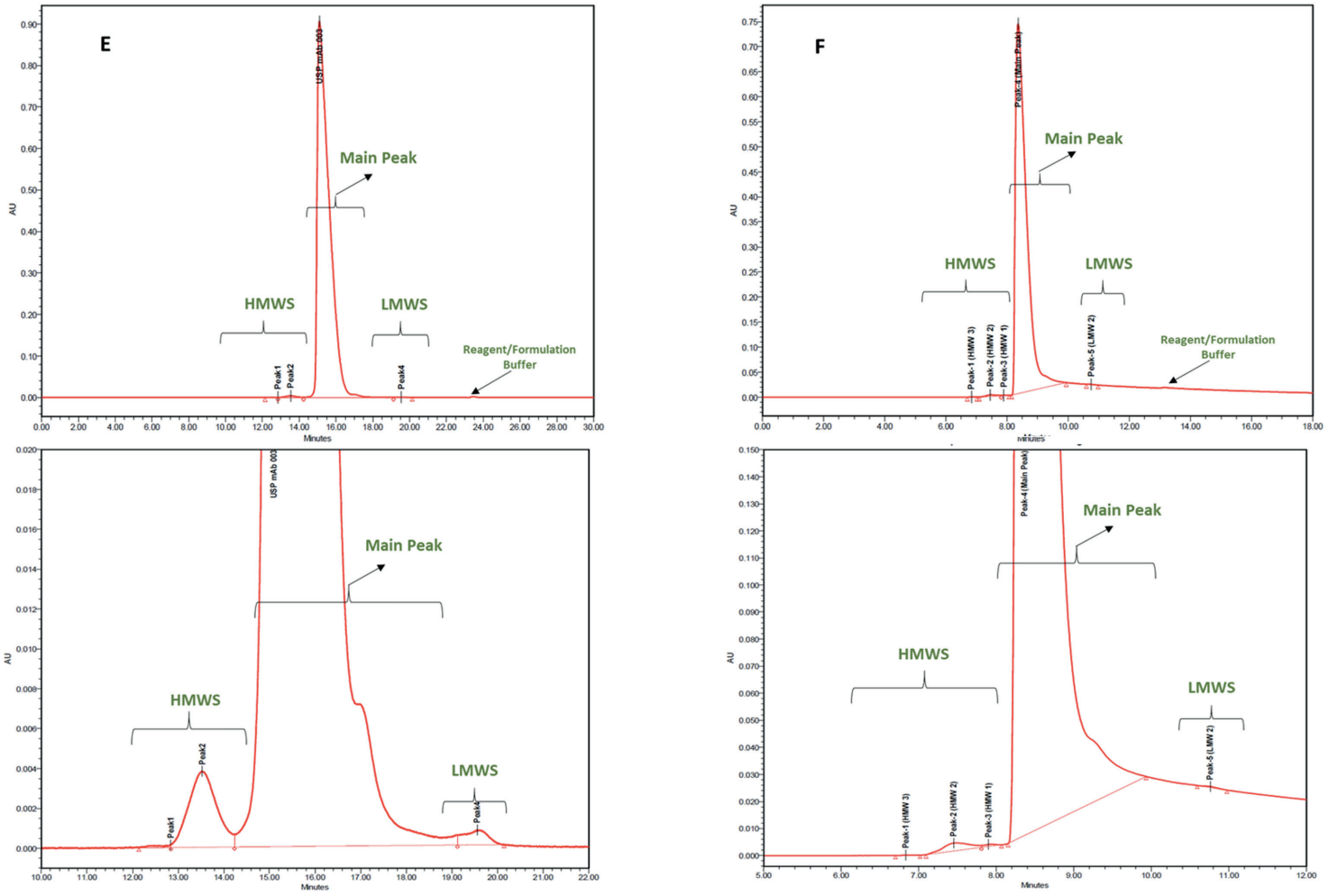

Figure 4: Representative Size Exclusion Chromatograms for USP mAb 003 Monoclonal IgG1 (E) SE-HPLC (F) SE-UHPLC. Top images show full scale while bottom images show zoomed scale.

Conclusion

This work, along with the recently revised General Chapter <129>, highlights an alternative SEC method to assess purity. The SE-UHPLC method allows for higher throughput and uses significantly less sample. These advantages have led to an increased interest in transitioning to UHPLC methods. The results from this study confirm that the two SEC methods from *USP* General Chapter <129>, SE-HPLC and SE-UHPLC, perform comparably when using USP mAb 001, 002, or 003.

The SE-UHPLC method does, however, require an advanced liquid chromatography instrument, because a traditional HPLC cannot withstand the high pressure demanded of the SE-UHPLC method. As with all compendial methods, a verification study following *USP* Chapter <1226> *Verification of Compendial Procedures* is required prior to implementation of the SE-UHPLC purity determination method in a quality control environment.

References

1. Pham NB, Meng WS. Protein aggregation and immunogenicity of biotherapeutics. Int J Pharm. 2020 585:119523
2. USP-NF General Chapter <129> - Analytical Procedures for Recombinant Therapeutic Monoclonal Antibodies
3. USP mAb 001, Monoclonal IgG1 Certificate
4. USP mAb 002, Monoclonal IgG1 Certificate
5. USP mAb 003, Monoclonal IgG1 Certificate
6. USP Technical Note - USP Monoclonal Antibody Reference Standard
7. USP Technical Note - Charge Variant Analysis of USP Monoclonal Antibody Reference Standards
8. Monoclonal IgG System Suitability Certificate

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